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SAFETY TESTING OF DENGUE-1 AND DENGUE-3 SEEDS FOR HUMAN CHALLENGES, UNATTENUATED; DENGUE VIRUS TYPE 4

PHASE REPORT

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Dengue Virus Type 4 (CARIB 341750), Challenge Seed, non-attenuated, Lot No. 2 of Jan 1986 was safety tested in accordance with the guidelines established by the FDA for live and inactivated vaccines, as stipulated in 21 CFR, Parts 610.11, 610.12, 610.30, 630.10-630.18 etc. All testing procedures were carried out following Good Laboratory Practices (GLP) regulations (21 CFR, Part 58).

However, the Tissue Culture Purity (Safety) tests were unsatisfactory due to the use of a hyperimmune rabbit antiserum that was subsequently found to readily hemagglutinate guinea pig RBC suggesting the presence in the serum of a hemagglutinating agent(s).

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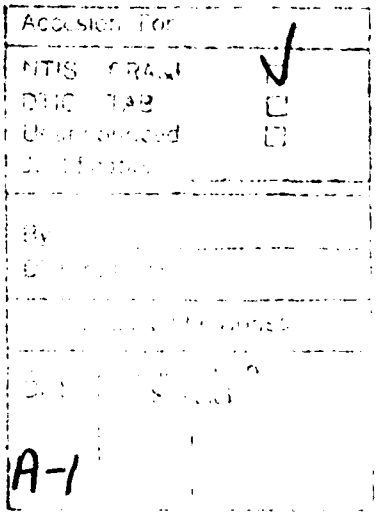
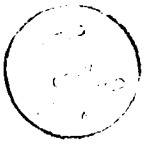
FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the Guide for the Care and Use of Laboratory Animals prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHHS, PHS, NIH Publications No. 85-23, Revised 1985).





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I. INTRODUCTION

The accompanying protocol is a description of the safety testing of a lot of dengue virus type 4 designated as:

Dengue Virus Type 4 (Carib 341750)
Challenge Seed

Utilizing the testing procedures herein described, this fluid is considered to have not passed satisfactorily all tests for safety including purity. The detailed records with respect to passage history, pool production, final product, virus characterization and subsequent safety testing may be found in the laboratory notebooks located at:

The Walter Reed Army Institute of Research (WRAIR), Bldg. 501, Washington, DC 20307-5100 - (Dr. Ken Eckels)

The Experimental Virus Vaccine Production Laboratory - Suite #500 - Flow Laboratories, Inc., McLean, VA - (Dr. Louis Potash)

All procedures performed at Flow Laboratories followed Good Laboratory Practices (GLP) regulations (21 CFR, Part 58) and were carried out in accordance with the guidelines established by the FDA for live and inactivated vaccines as found in 21 CFR, Parts 610.11, 610.12, 610.30, 630.10 - 630.18, etc. These procedures are detailed in the following SOPs and recorded on the indicated VVPL Forms:

SOP No.:	400.002 -	Issued	25 Feb 1980,	Revised	18 Feb	1986
	400.004 -	"	25 Feb 1980,	"	18 Feb	1986
	400.005 -	"	25 Feb 1980,	"	18 Feb	1986
	400.006 -	"	25 Feb 1980,	"	18 Feb	1986
	400.007 -	"	25 Feb 1980,	"	18 Feb	1986
	400.008 -	"	12 Apr 1984,	"	18 Feb	1986
	400.009 -	"	3 May 1984,	"	18 Feb	1986
	500.001 -	"	29 Oct 1980,	"	18 Feb	1986
	500.002 -	"	29 Oct 1980,	"	18 Feb	1986
	500.008 -	"	13 Jan 1981,	"	3 Mar	1986
	500.009 -	"	23 Feb 1981,	"	3 Mar	1986
VVPL FORM	#001 -	Issued	25 Feb 1981,	Revised	2 Mar	1984
	003 -	"	3 Apr 1984			
	004 -	"	16 Jan 1981,	"	21 Mar	1984
	008 -	"	29 Oct 1980,	"	3 May	1984
	016 -	"	15 Jan 1981,	"	13 July	1984
	017 -	"	16 Jan 1981,	"	13 Jan	1986
	019 -	"	8 Oct 1984			
	023 -	"	19 Feb 1986			

II. SYNOPSIS

A.	Virus Strain:	Dengue Virus Type 4 (Carib 341750) Challenge Seed
B.	Live Virus Pool Designation:	MFG Date: Jan 1986, LOT No. 2
C.	Treatment/Handling	Freeze-Dried: Rehydrate to 3 ml with Sterile Distilled Water

D. Safety Tests on Crude Harvest Fluids:

1. Sterility: Fluid Thioglycollate (FTM), Tryptone Soya Broth (TSB), Lowenstein-Jensen Egg Medium, Mycoplasma

a. Virus Infected Fluid	(41 ml)	Growth in TSB*
b. Control Fluid (TCF)	(52 ml)	No Growth

2. Tissue Culture Identity and Purity (Safety): AGMK, PHA, FRhL-2, PRK, and Flow 5000.

a. Virus Infected Fluid	(30 ml)	Unsatisfactory**
b. Control Fluid (TCF)	(50 ml)	Satisfactory

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* Growth observed only in TSB cultures - identified as micrococcus species.

** Morphological changes observed in primary and subpassaged AGMK cultures with complete inhibition of the Coxsackie A-9 challenge virus in those tubes inoculated with 14-day harvest fluid derived from flask initially inoculated with serum/virus mixture. In addition, all flasks inoculated with serum/virus mixture and their respective subpassages into homologous tube cultures when tested for hemadsorption with guinea pig RBC exhibited clumping and subsequent hemagglutination (no hemadsorption). A follow-up testing of the specific rabbit antiserum gave a hemagglutination titer of $\geq$1:320 when unheated and 1:64 after heat-inactivation (56°C/30 min.). Furthermore, all 14-day harvests gave hemagglutination titers of >1:64 when assayed before and after heat-inactivation. The apparent presence of a hemagglutinating agent(s) in the rabbit antiserum has clouded the interpretation of the tissue culture purity (safety) tests.

3. Animal Safety:

a.	Adult Mice: Intracerebral and I.P.		
(1)	Virus Infected Fluid	(11 ml)	Satisfactory
(2)	Control Fluid (TCF)	(11 ml)	Satisfactory
b.	Suckling Mice: Intracerebral and I.P.		
(1)	Virus Infected Fluid	(2.5 ml)	All Died
(2)	Virus Fluid Neutralized	(2.5 ml)	Questionable***
(3)	Control Fluid (TCF)	(2.5 ml)	Satisfactory
c.	Guinea Pigs: Intracerebral and I.P.		
(1)	Virus Infected Fluid	(15.5 ml)	Satisfactory
(2)	Control Fluid (TCF)	(15.5 ml)	Satisfactory
d.	Rabbits: Intradermal and Subcutaneous		
(1)	Virus Infected Fluid		Not Done
(2)	Control Fluid (TCF)	(20 ml)	Satisfactory

E. Final Product Testing:

1.	Microbial Sterility: Fluid Thioglycollate & Soybean-Casein Digest Media	(10 x 3 ml vials)	No Growth
2.	Reverse Transcriptase:	(1 ml)	No RT Enzyme
3.	General Safety:		
a.	Mice: I.P.	(2 x 0.5 ml)	Satisfactory
b.	Guinea Pigs: I.P.	(2 x 5.0 ml)	Satisfactory

*** Although only 9 of 20 sucklings survived the initial 14-day incubation period, all 20 sucklings inoculated with the emulsified tissue from these 9 mice survived the final 14-day blind passage with no signs of illness or distress.

III. DETAILED SUMMARY RELATING TO THE SAFETY TESTING OF A LOT OF DENGUE VIRUS TYPE 4 (CARIB 341750), NON-ATTENUATED CHALLENGE SEED: PROPAGATED IN DBS-FRhL-2 CELL CULTURES

A. Inocula

On January 15, 1988, the following frozen materials were obtained for testing from Dr. K. Eckels, Contracting Officer's Representative, at the Walter Reed Army Institute of Research (WRAIR), Bldg. 501, Washington, DC 20307-5100:

1. Dengue-4 (Carib 341750) challenge seed, day 5 harvest, unclarified of 15 Jan 1986: 10 x 10 ml vials

2. Dengue-4 (Carib 341750) control fluids, day 5 harvest, unclarified of 15 Jan 1986: 9 x 20 ml vials

3. Dengue-4, non-attenuated, Carib 341750, freeze-dried Final Product, Mfg. Date: Jan 1986, LOT No.2: 15 x 3ml vials

On February 25, 1988, the following antiserum was obtained from Dr. K. Eckels:

4. Rabbit Antiserum: Dengue-4 (CAREC 814669), smb 6 of 18 Feb 88: 3 x 20 ml vials and 1 x 25 ml vial.

On arrival in this laboratory, the materials were stored as follows: Items #1 and #2 at -70°C, or below; Items #3 and #4 at -20°C, or below.

B. Safety Testing Procedures and Results on the Crude, Unclarified Harvest Fluids (SOP No.: 500.008)

1. Microbial Sterility - (VVPL FORM #011)

Aliquots of the bulk frozen fluids were thawed and tested for microbial sterility as follows:

a. Fluid Thioglycollate Medium - FTM - (LOT #35045215): Each of 5 culture tubes (9-10 ml medium per tube) was inoculated with 1 ml volumes of the crude virus fluid and each of 10 culture tubes was inoculated with 1 ml volumes of the crude control fluid. An additional 10 cultures were included as uninoculated controls. All cultures were vortex mixed and incubated at 31°C (± 1°C) for 21 days with periodic examination for evidence of growth. No growth was observed in any of the 25 culture tubes.

b. Tryptone Soya Broth - TSB - (LOT #35060235): Each of 5 culture culture tubes (9-10 ml medium per tube) was inoculated with 1 ml volumes of the crude virus fluid and each of 10 culture tubes was inoculated with 1 ml volumes of the crude control fluid. An additional 10 cultures were included as uninoculated controls. All cultures were vortex mixed and incubated at 22°C (± 2°C) for 21 days with periodic examination for evidence of growth. Growth was observed in all 5 tubes inoculated with virus on the 18th day of incubation with the contaminant identified as a micrococcus species. No growth was observed in the remaining 20 culture tubes. Due to the low volume of virus fluid available for other testing, no attempt was made to repeat this sterility test in TSB cltures.

c. Lowenstein-Jensen Egg Medium (BBL - Lot #J8CZKK): Each of 10 culture tubes was inoculated with 0.5 ml of the crude virus fluid and each of 10 culture tubes was inoculated with 0.5 ml of the crude control fluid. Ten additional culture tubes were included as uninoculated controls. All cultures were incubated at 37°C — horizontally for the first 24 hours and then vertically for the remainder of the 8-week observation period. Cultures were examined periodically for growth over this 8-week period. No growth was observed in any of the 30 cultures.

The results of the above described Microbial Sterility Assays are summarized in Table I.

d. Mycoplasma Sterility: These assays were performed by the Flow Laboratories' Mycoplasma Testing Laboratory and included both the routine PPLO agar and broth assays and the specific test for the detection of M. hyorhinis. Samples (1 x 5 ml and 1 x 1 ml of the crude virus fluid; 1 x 25 ml and 1 x 2 ml of the control fluid) were submitted for testing. All samples were reported to be negative for mycoplasmas. A copy of this report is appended to this Protocol - (Appendixes - 1 & 2).

2. Identity in Tissue Culture (Serum-Neutralization) - (VVPL FORM #015)

No attempt was made to identity the crude virus pool in tissue cultures.

3. Purity (Safety) in Tissue Cultures - (VVPL FORM #016)

a. Tissue Cultures: "Fully" sheeted flask or roller tube cell cultures were prepared by laboratory personnel. Cultures were maintained on Medium MEM containing 5 to 10% fetal bovine serum (heat-inactivated) plus antibiotics: gentamicin @ 100 mcg/ml; neomycin @ 50 mcg/ml; and amphotericin B (I.V.) @ 2.5 mcg/ml. Cultures were inoculated, refed and subpassaged as indicated below. The following tissue culture systems were utilized:

(1) Tertiary African Green Monkey Kidney (AGMK) ..... MEM + 5% serum
(2) Primary Human Amnion (PHA) ..... MEM + 10% serum
(3) Fetal Rhesus Lung (FRhL-2) ..... MEM + 5% serum
(4) Primary Rabbit Kidney (PRK) ..... MEM + 5% serum
(5) Whole Human Embryo Fibroblast (Flow 5000) ..... MEM + 5% serum

b. General Testing Procedures

(1) Crude Virus Fluid

(a) Primary Flask Cultures: Equal 5 ml volumes of the bulk crude virus fluid and of a 1:2 dilution of the rabbit immune serum (Den-4, CAREC 814669, smb 6) were well mixed and incubated at 37°C (water bath) for 2 hours. Due to the small volume of crude virus fluid available, only 5 ml of the virus fluid was tested per tissue culture system wherein 1 x 75 cm^2 flask per tissue culture system was inoculated with 10 ml of this serum-virus mixture. Flasks contained approximately 25 ml of maintenance medium at the time of inoculation. Cultures were incubated at 35°C (37°C for PHA) for 14 days with periodic microscopic examination for any signs of CPE and/or cellular degradation. When necessary to maintain the integrity of the cell films, cultures were refed with 35 ml of fresh medium.

(b) Secondary Tube Subcultures: On the 14th day of incubation, the primary cultures were re-examined microscopically and the fluids harvested individually and treated with the specific immune serum - 1.0 ml per harvest. In addition, to each individual harvest was added: 0.1 ml gentamicin (50 mg/ml); 1 ml penicillin-streptomycin solution (5000 units/ml and 5000 mcg/ml, respectively); and 10% of 10X SPG* (v/v). Following mixing, the fluids were incubated at room temperature for 60 minutes and then subpassed into homologous roller tube cultures - 0.5 ml of each harvest into each of 20 tubes. The remainder of the harvest fluids was saved and stored at -75°C, or below. All primary cultures were tested for hemadsorption by the addition of 0.1% guinea pig RBC (in PBS) and incubation at 4°C for a minimum of 30 minutes. All cultures were negative for hemadsorption but all were positive for clumping and subsequent hemagglutination.

Tube cultures (refed with 2 ml of maintenance medium prior to inoculation) were incubated at 35°C (37°C for PHA) for 13-14 additional days. When necessary to maintain the integrity of the cell films, cultures were refed with 2 ml of fresh medium. Cultures were examined microscopically at periodic intervals and at the end of the incubation period for any signs of CPE. After final examination, tubes were divided - depending on the specific cell system - for additional testing:

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* 10X SPG: sucrose, 2.18 M; KH_2PO_4, 0.038 M; K_2HPO_4, 0.072 M; potassium glutamate, 0.049 M.

AGMK, PHA, FRhL-2 and Flow 5000 Tube Cultures: These were divided into 3 groups as follows:

1/4th tested for the presence of hemadsorbing agents,
1/4th fixed and stained with a solution of 5% glutaraldehyde + 1:10 giemsa stain and examined microscopically for any CPE,
1/2 Challenged with Coxsackie A-9 virus (0.2 ml per tube at dilutions noted in the tables) for the detection of non-CPE producing agents and/or latent agents via the interference phenomenon.

PRK Tube Cultures: These were equally divided into 2 groups:

1/2 tested for the presence of hemadsorbing agents,
1/2 fixed and stained with the glutaraldehyde-giemsa stain solution and examined microscopically for any CPE.

No challenge studies were carried out with the Coxsackie A-9 virus since this virus does not produce any discernible CPE in this tissue culture system.

(2) Crude Control Fluid

Equal 10 ml volumes of the crude control fluid and the indicated maintenance medium were well mixed and incubated at 37°C (water bath) for 2 hours. A total of 10 ml of the control fluid was tested per tissue culture system wherein each of 2 x 75 cm^2 flasks per tissue culture system was inoculated with 10 ml of the above mixture. Cultures were handled in a manner similar to that described above for the crude virus fluid except that immune serum was not included.

(3) Uninoculated Cell Lot Controls

Two x 75 cm^2 flasks per tissue culture system were included as uninoculated cell lot controls and were handled in a manner similar to that described above for the crude virus fluid except that immune serum was not included. In addition, an appropriate number of uninoculated roller tube cultures were included as cell lot controls for the secondary tube subcultures.

In all challenge studies, 1 to 4 culture tubes per set were left unchallenged to serve as controls to the challenge virus.

The results of these in vitro Tissue Culture Purity (Safety) tests are summarized in Tables II-A through -E.

4. Animal Safety Tests - (VVPL FORM #004)

a. Adult Rabbits - Test for B-virus and other adventitious agents (SOP No.: 400.004)

Due to the insufficient volume of crude virus fluid available for testing and after consulting with Dr. Eckels, it was determined that no rabbits would be inoculated with this fluid. However, since there was a sufficient volume of control fluid available, it was decided to proceed with the assay in rabbits using only the crude control fluid. Each of 2 New Zealand white rabbits (1500-2500 grams each) was inoculated intradermally in multiple sites with a total of 1.0 ml and subcutaneously with 9.0 ml with this fluid. In addition, the left cornea was scratched and 0.03 ml of the crude control fluid was applied. One additional rabbit was included as an uninoculated control. All rabbits were observed daily for a total of 28 days for deaths and/or signs of lesions at sites of inoculation and for any signs of illness or distress. All rabbits remained healthy and none exhibited any signs of illness or distress or lesions at the sites of inoculation. This test in adult rabbits with the crude control fluid was considered satisfactory.

b. Adult Mice - Test for adventitious agents - (SOP No. 400.005)

Each of 20 adult CD-1 mice (15-20 grams each) was inoculated intracerebrally with 0.03 ml and intraperitoneally with 0.5 ml of the un-neutralized crude virus fluid and each of 20 adult CD-1 mice was similarly inoculated with the crude control fluid. An additional 10 mice were included as uninoculated controls. The mice were observed daily for deaths and/or signs of illness or distress over a 4 week period. All mice (inoculated as well as controls) remained healthy and survived the entire 28-day observation period with no evidence of lymphocytic choriomeningitis virus infection or of any other virus infection. This test in adult mice was considered satisfactory.

c. Suckling Mice - Test for adventitious agents - (SOP No.: 400.005)

Three groups of 20 newborn CD-1 mice from mixed litter (10 per mother - less than 24 hours old) were inoculated intracerebrally with 0.01 ml and intraperitoneally with 0.1 ml as follows: group (a) with un-neutralized crude virus fluid; group (b) with neutralized virus fluid (equal parts of the undiluted antiserum and of the crude virus fluid); and group (c) with the crude control fluid. An additional litter of 10 sucklings was included to serve as uninoculated controls. All sucklings were observed daily for 14 days for deaths and/or signs of illness or distress. Of the sucklings in group (a), all were found dead or cannibalized within 13 days: 3 within the first 24 hours, 8 within 48 hours, 2 on day 11, 5 on day 12 and 2 on day 13. Of the sucklings in group (b), a total of 11 were found cannibalized: 2 on day 11, 3 on day 12, 5 on day 13 and 1 on day 14. No deaths were recorded in group (c) or in the uninoculated control litter over this initial 14-day observation period.

On the 14th day, single pools were prepared of the emulsified tissue (minus skin and viscera) of the following groups: b) neutralized virus inoculated sucklings [9]; c) control fluid inoculated sucklings [20]; and d) uninoculated controls [10]. A blind passage into newborn CD-1 mice was made of each of the 3 pools via the intracerebral and intraperitoneal routes: the individual pools from the inoculated sucklings (b and c) into each of 20 newborns and the pool from the uninoculated control sucklings (d) into 10 newborns. An additional litter of 10 sucklings was included as uninoculated controls (e) for this blind passage. All sucklings were observed daily for 14 days for deaths and/or signs of illness or distress. There were no deaths and none of the sucklings exhibited any signs of illness or distress over this final 14-day observation period.

Although only 9 of 20 (45%) of the sucklings inoculated with the neutralized virus survived the initial 14-day observation period, all 20 sucklings (100%) inoculated with their emulsified tissue pool survived without exhibitng any evidence of a transmissible agent or of Coxsackie virus infection or of any viral infection. However, due to the excessive number of deaths/cannibalizations observed in the virus inoculated sucklings within the initial 14-day observation period, it would be difficult to consider this test in suckling mice to be satisfactory.

d. Adult Guinea Pigs - (SOP No.: 400.006)

Test for M. tuberculosis: Each of 3 adult guinea pigs (Hartley Strain, virus free, 350-450 grams each) was inoculated intracerebrally with 0.1 ml and intraperitoneally with 5 ml of the un-neutralized crude virus fluid, and each of 3 guinea pigs was similarly inoculated with the crude control fluid. An additional 3 guinea pigs were included as uninoculated controls. All pigs were observed daily for a period of 6 weeks for deaths and/or any signs of illness or distress. Commencing on day 21, daily rectal temperatures (LED digital thermistor thermometer) were taken and recorded (± 0800 hrs) for all guinea pigs until time of sacrifice. The average temperatures (^{o}C) for the 3 groups of guinea pigs were: 38.49, 38.51, and 38.60 for the virus fluid inoculated; 38.36, 38.40 and 38.57 for the control fluid inoculated; and 38.47, 38.52 and 38.61 for the uninoculated controls. There were no significant rises indicative of either bacterial or viral infection. All guinea pigs appeared healthy and survived the entire 42-day observation period at which time they were necropsied following euthanasia with Halathane. Inspection of the abdominal and thoracic cavities indicated no gross pathological changes. This test in guinea pigs was considered satisfactory.

The results of these in vivo Animal Safety Tests are summarized in Table III - A and - B.

C. Final Product Testing and Results - (SOP No.: 500.009)

1. Microbial Sterility

A total of 10 x 3 ml vials of the freeze-dried final virus product was submitted to Ben-Venue Laboratories, Inc., for microbial sterility testing via the Membrane Filtration Method in Fluid Thioglycollate and Fluid Soybean-Casein Digest Media. No growth was reported and a copy of this report is appended to this Protocol - (Appendixes - 3 & 4).

2. Reverse Transcriptase - Assay for the detection of RNA-dependent DNA-polymerase activity

The assay for Reverse Transcriptase was performed by Dr. Allan Tereba at the St. Jude Children's Research Hospital, Memphis, TN. A 1.0 ml sample of the reconstituted freeze-dried virus fluid and a 2 ml sample of the clarifed (centrifuged) control fluid were submitted for assay. Both samples were reported to be negative for the RT Enzyme and a copy of this report is appended to this Protocol - (Appendix - 5).

3. General Safety Test - (SOP No.: 400.002 - VVPL FORM #001)

Each of 2 overtly healthy CD-1 mice (less than 22 grams each) and each of 2 overtly healthy guinea pigs (Hartley Strain, virus free - less than 400 grams each) were inoculated intraperitoneally with 0.5 ml and 5 ml, respectively, of the reconstituted freeze-dried final virus product. Two additional animals of each species were included as uninoculated controls. All animals were weighed prior to inoculation and on day 7 post inoculation. All animals were observed daily over this 7-day period for deaths and/or signs of illness or distress - none were noted. All animals remained healthy and all exhibited weight gains. This test was considered satisfactory. The results of these General Safety Tests are summarized in Table IV.

Table I. Microbial Sterility Test Results on the Crude Dengue-4 Virus (Carib 341750) Challenge Seed

Culture Medium	No.	Vol. per culture (ml)	Temperature	Date On Test	Date Off Test	Results
Fluid Thioglycollate (FTM) LOT #35045215	10	-----	31°C (±1°C)	3/10/88	3/31/88	No Growth
Virus Infected Fluid	5	1.0		|	|	No Growth
Control Fluid	10	1.0		3/10/88	3/31/88	No Growth
Tryptone Soya Broth (TSB) LOT #35060235	10	-----	22°C (±2°C)	3/10/88	3/31/88	No Growth
Virus Infected Fluid	5	1.0		|	3/28/88	D18 5/5 Growth*
Control Fluid	10	1.0		3/10/88	3/31/88	No Growth
Lowenstein-Jensen Egg Medium - LOT #J8CZKK	10	-----	36.5°C (±1°C)	3/15/88	5/10/88	No Growth
Virus Infected Fluid	10	0.5		|	|	No Growth
Control Fluid	10	0.5		3/15/88	5/10/88	No Growth

* Contaminant identified as micrococcus species.

Table II. Tissue Culture Purity (Safety) Test Results on the Crude Dengue-4 Virus (Carib 341750) Challenge Seed

A. Tertiary African Green Monkey Kidney (AGMK) - Initial Assay

	Initial Flasks			0.5 ml per tube Passage #1						
	Lot # 275 (2133)			Lot # 287 (2133)						
	Day 14			Day 14 + 14 = 28						
							Coxsackie A-9 Challenge*			
Material Tested	CPE	Hads	Stain	CPE	Hads	Stain	10^{-3}	10^{-4}	10^{-5}	10^{-6}
	**	a	***	#	a	***				
Virus/Serum Mixture	1/1	1/1	1/1	20/20	5/ 5	5/ 5	0/2	0/2	0/2	0/2
Control Fluid (TCF)	0/2	0/2	0/2	0/40	0/10	0/10	4/4	4/4	4/4	1/4
Control - (1)	0/2	0/2	0/2	0/40	0/10	0/10	4/4	4/4	4/4	3/4
Control - (2)				0/48	0/12	0/12	5/5	5/5	5/5	3/5

* Coxsackie A-9 Challenge Results based on a 3-day incubation at 35°C. All tubes refed with 2 ml of fresh medium prior to challenge. Complete inhibition of Coxsackie A-9 challenge virus by virus/serum mixture series.

** Flask exhibited non-descript CPE.

*** Staining of films both in flask and tubes confirmed morphological changes in these cultures in contrast to the control fluid inoculated and uninoculated controls.

a Rather than hemadsorption, flask and tubes indicated clumping of RBC with subsequent hemagglutination. When the antiserum alone was assayed for hemagglutination vs guinea pig RBC, titers of 1:64 when heat-inactivated (56°/30 min.) and ≥1:320 when unheated were obtained. Furthermore, when the 14-day harvest was assayed for hemagglutination vs guinea pg RBC, a titer of >1:64 was obtained both before and after heat-inactivation. These results suggested that the antiserum contained an agent capable of hemagglutinating guinea pig RBC.

\# On day 20 (days 14 + 6), tubes initially exhibited morphological changes, which by day 24 (days 14 + 10) were more extensive. On day 24, tube-to-tube subpassage performed into same lot of tubes (#287) with the addition of 0.5 ml immune serum per tube. The initial tubes when hemadsorbed exhibited negative hemadsorption but positive hemagglutination.

Table II. Tissue Culture Purity (Safety) Test Results on the Crude Dengue-4 Virus (Carib 341750) Challenge Seed

A. Tertiary African Green Monkey Kidney (AGMK) - Repeat Assay

	Initial Flasks			0.5 ml per tube Passage #1						
	Lot # 316 (2133)									
	Day 6									
							Coxsackie A-9 Challenge*			
Material Tested	CPE	Hads	Stain	CPE	Hads	Stain	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Virus alone	1/1	0/1	1/1	ND						
Control Fluid (TCF)	0/2	0/2	0/2	ND						
Control - (1)				ND						

This study was attempted wherein the virus was not treated with any antiserum but incubation was at 37°C in an effort to inhibit or slow down Dengue-4 virus replication. However, on on day 3, film exhibited morphological changes which by day 6 completely destroyed film. All other cultures indicated no morphological changes. None of the cultures were positive for hemadsorption or hemagglutination. Study terminated on day 6.

Table II. Tissue Culture Purity (Safety) Test Results on the Crude Dengue-4 Virus (Carib 341750) Challenge Seed

B. Primary Human Amnion (PHA)

	Initial Flasks			0.5 ml per tube Passage #1						
	Lot # 265			Lot # 279						
	Day: 14			Day: 14 + 14 = 28						
							Coxsackie A-9 Challenge*			
Material Tested	CPE	Hads	Stain	CPE **	Hads	Stain	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Virus/Serum Mixture	0/1	1/1 [a]	0/1	0/20	5/ 5 [a]	0/ 5	2/2	2/2	2/2	1/2
Control Fluid (TCF)	0/2	0/2	0/2	0/40	0/10	0/10	4/4	4/4	4/4	4/4
Control - (1)	0/2	0/2	0/2	0/40	0/10	0/10	4/4	4/4	4/4	2/4
Control - (2)				0/60	0/12	0/12	8/8	8/8	8/8	8/8

* Coxsackie A-9 Challenge Results based on a 5-day incubation at 37°C. All tubes refed with 2 ml of fresh medium prior to challenge.

** On day 24 (14 + 10), all tubes were refed with 2 ml of fresh medium.

a Both the original flask and those tubes inoculated with the harvest from this flask were negative for hemadsorption but positive for clumping and subsequent hemagglutination. When the antiserum alone was assayed for hemagglutination vs guinea pig RBC, titers of 1:64 when heat-inactivated (56°/30 min.) and $\geq$1:320 when unheated were obtained. Furthermore, when the original 14-day harvest was assayed for hemagglutination vs guinea pg RBC, a titer of >1:64 was obtained both before and after heat-inactivation. These results suggested that the antiserum contained an agent capable of hemagglutinating guinea pig RBC.

Table II. Tissue Culture Purity (Safety) Test Results on the Crude Dengue-4 Virus (Carib 341750) Challenge Seed

C. Fetal Rhesus Lung (FRhL-2)

	Initial Flasks			0.5 ml per tube Passage #1						
	Lot # 271 p17			Lot # 291 p21A						
	Day 14			Day 14 + 13 = 27						
							Coxsackie A-9 Challenge*			
Material Tested	CPE	Hads	Stain	CPE ***	Hads	Stain	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Virus/Serum Mixture	1/2 **	1/2 [a]	1/2 [b]	0/40	5/10 [a]	0/10	4/4	4/4	3/4	2/4
Control Fluid (TCF)	0/2	0/2	0/2	0/40	0/10	0/10	4/4	4/4	3/4	2/4
Control - (1)	0/2	0/2	0/2	0/40	0/10	0/10	4/4	4/4	3/4	1/4
Control - (2)				0/42	0/12	0/12	4/4	4/4	4/4	1/4

* Coxsackie A-9 Challenge Results based on a 7-day incubation at 35°C in FRhL-2 cells followed by a tube-to-tube subpassage into AGMK tubes and an additional 2-day incubation at 35°C. Results reflect CPE as observed in AGMK tubes.

** On day 7, single flask exhibited morphological changes and fluid supassaged to new flask of same cell lot. Original flask refed with fresh medium. To both flasks was added 2 ml of the undiluted immune rabbit serum. On day 14, original flask exhibited marked degeneration.

*** On day 20 (day 14 + 6), all tubes were refed with 2 ml of fresh medium. No cellular degeneration was detected in the tube cultures.

a Both the original flask and those tubes inoculated with the harvest from this flask were negative for hemadsorption but positive for clumping and subsequent hemagglutination. When the antiserum alone was assayed for hemagglutination vs guinea pig RBC, titers of 1:64 when heat-inactivated (56°/30 min.) and ≥1:320 when unheated were obtained. Furthermore, when the original 14-day harvest was assayed for hemagglutination vs guinea pg RBC, a titer of >1:64 was obtained both before and after heat-inactivation. These results suggested that the antiserum contained an agent capable of hemagglutinating guinea pig RBC.

b Staining confirmed the marked morphological changes initially observed in the original flask inoculated with the serum/virus mixture.

Table II. Tissue Culture Purity (Safety) Test Results on the Crude Dengue-4 Virus (Carib 341750) Challenge Seed

D. Primary Rabbit Kidney (PRK)

	Initial Flasks			0.5 ml per tube Passage #1		
	Lot # 264			Lot # 278		
	Day: 14			Day: 14 + 14 = 28		
Material Tested	CPE	Hads	Stain	CPE	Hads	Stain
Virus/Serum Mixture	0/1	1/1[a]	0/1	0/20	10/10[a]	0/10
Control Fluid (TCF)	0/2	0/2	0/2	0/40	0/20	0/20
Control - (1)	0/2	0/2	0/2	0/40	0/20	0/20
Control - (2)				0/24	0/12	0/12

a Both the original flask and those tubes inoculated with the harvest from this flask were negative for hemadsorption but positive for clumping and subsequent hemagglutination. When the antiserum alone was assayed for hemagglutination vs guinea pig RBC, titers of 1:64 when heat-inactivated (56°/30 min.) and >1:320 when unheated were obtained. Furthermore, when the original 14-day harvest was assayed for hemagglutination vs guinea pg RBC, a titer of >1:64 was obtained both before and after heat-inactivation. These results suggested that the antiserum contained an agent capable of hemagglutinating guinea pig RBC.

Table II. Tissue Culture Purity (Safety) Test Results on the Crude Dengue-4 Virus (Carib 341750) Challenge Seed

E. Whole Human Embryo Fibroblasts (Flow 5000)

	Initial Flasks			0.5 ml per tube Passage #1						
	Lot # 271 p17			Lot # 291 p21						
	Day 14			Day 14 + 14 = 28						
							Coxsackie A-9 Challenge*			
Material Tested	CPE	Hads	Stain	CPE	Hads	Stain	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Virus/Serum Mixture	0/1	1/1[a]	0/1	0/20	0/ 5[a]	0/ 5	2/2	2/2	2/2	0/2
Control Fluid (TCF)	0/2	0/2	0/2	0/40	0/10	0/10	4/4	4/4	4/4	3/4
Control - (1)	0/2	0/2	0/2	0/40	0/10	0/10	4/4	4/4	3/4	1/4
Control - (2)				0/60	0/12	0/12	8/8	8/8	8/8	3/8

* Coxsackie A-9 Challenge Results based on a 3-day incubation at 35°C. All tubes refed with 2 ml of fresh medium prior to challenge.

a Both the original flask and those tubes inoculated with the harvest from this flask were negative for hemadsorption but positive for clumping and subsequent hemagglutination. When the antiserum alone was assayed for hemagglutination vs guinea pig RBC, titers of 1:64 when heat-inactivated (56°/30 min.) and ≥1:320 when unheated were obtained. Furthermore, when the original 14-day harvest was assayed for hemagglutination vs guinea pg RBC, a titer of >1:64 was obtained both before and after heat-inactivation. These results suggested that the antiserum contained an agent capable of hemagglutinating guinea pig RBC.

Table III - A. Animal Safety Tests Results on the Crude Dengue-4 Virus (Carib 341750) Challenge Seed

Animal Species	Inoculum	Vol. (ml)	Route	No.	Lesions, Illness or Deaths over 4 to 6 Week Period	Comments
Adult Rabbits (1500-2500 gms)	Virus Pool Un-neutralized	10 x 0.1 1 x 9.0 1 x 0.03	I.D. S.Q. L.Cornea	None	There were no lesions at sites of inoculation. No deaths nor signs of illness or distress.	Test Satisfactory
	Control Fluid (TCF)	10 x 0.1 1 x 9.0 1 x 0.03	I.D. S.Q. R. Cornea	2		
	None	———	——	1		
Adult Mice (15-20 grams)	Virus Pool Un-neutralized	0.03 0.50	I. Cer. I.P.	20	No deaths nor signs of illness or distress recorded.	Test Satisfactory
	Control Fluid (TCF)	0.03 0.50	I. Cer. I.P.	20		
	None	——	——	10		
Suckling Mice (< 24 hours)	Virus Pool Un-neutralized	0.01 0.10	I.Cer. I.P.	20	All 20 sucklings dead/cannibalized by Day 13.	Test of Questionable Validity
	Virus Pool Neutralized	0.01 0.10	I.Cer. I.P.	20	11 of 20 sucklings dead/cannibalized between days 11-14	
	Control Fluid (TCF)	0.01 0.10	I.Cer. I.P.	20	No other deaths nor signs of illness or distress over this initial 14-day period.	
	None	——	——	10		
	Dl4 Blind Passage (VP-N)	0.01 0.10	I. Cer. I.P.	20	No deaths nor signs of illness or distress over this final 14-day period.	100% survival of sucklings inoculated with tissue emulsions. No evidence of a transmissible agent or of any viral infection.
	Dl4 Blind Passage (TCF)	0.01 0.10	I. Cer. I.P.	20		
	Dl4 Blind Passage (None)	0.01 0.10	I.Cer. I.P.	10		
	Dl4 - None	——	——	10		

Table III - B. Animal Safety Tests Results on the Crude Dengue-4 Virus (Carib 351750) *Challenge Seed*

Animal Species	Inoculum	Vol. (ml)	Route	No.	Lesions, Illness or Deaths over 4 to 6 Week Period	Comments
Adult Guinea Pigs (350-450 gms)	Virus Pool Un-neutralized	0.10 5.00	I. Cer. I.P.	3	No deaths nor signs of illness or disease. Daily rectal temperatures taken over last 3 weeks of observation (± 0800 hrs) were within normal ranges.	
	Control Fluid (TCF)	0.10 5.00	I.Cer. I.P.	3		
	None	—	—	3		

Code	GP#	Mean Temp. (°C)	Temp. Range (°C)
VP-1	712	38.49	38.2 - 38.8
VP-2	713	38.60	38.4 - 38.9
VP-3	714	38.51	38.3 - 38.8
TCF-1	715	38.36	38.0 - 38.6
TCF-2	716	38.57	38.2 - 38.8
TCF-3	717	38.40	38.0 - 38.8
C-1	718	38.61	38.3 - 38.9
C-2	719	38.47	38.2 - 38.7
C-3	720	38.52	38.0 - 39.0

Table IV. General Safety Test Results on the Final Product of Dengue-4 Virus (Carib 341750) Challenge Seed

Animal Species	Inoculum	Vol. (ml)	Tag #	Weight in Grams Day 0	Weight in Grams Day 7	Weight Gain/ (Loss) in Grams
Mice	Dengue-4	0.5	309	18.5	27.2	8.7
			310	20.8	29.8	9.0
	None	---	313	21.6	27.7	6.1
			314	20.8	25.5	4.7
Guinea Pigs	Dengue-4	5.0	721	383.0	422.6	39.6
			722	351.0	409.5	58.5
	None	---	725	345.1	387.5	42.4
			726	351.5	400.5	49.0

APPENDIX [illegible] 1

9 May 1988

Dr. Louis Potash
Flow Laboratories, Inc.
McLean, VA 22102

RE: Contract #833/8342

Dear Dr. Potash:

Your four samples, Dengue-4 Virus, Dengue-4 TCF, Hepatitis-A-Virus and Hepatitis A-TCF, which you submitted for the presence of mycoplasma hyorhinis using direct immuno-florescence staining, the DNA Hoechst stain and agar testing were found to be negative.

Sincerely,

Jim Quartey

JQ:kk

Flow Laboratories, Inc
7655 Old Springhouse Road, McLean, Virginia 22102, USA
Tel: (703) 893-5925 Fax: (703) 356-0774 Telex: 440543 FGI UI

MYCOPLASMA TEST RECORD SHEET

Culture Medium	LOT #	No. ml Tested		Date		Results
		Aerobic	Anaerobic	On Test	Off Test	
Virus Fluid - LOT # DENGUE-4 VIRUS MYC# 014						
PPLO Agar	870915	.2	.2	4/5/88	4/20/88	NEGATIVE
PPLO Broth	871015	5.0	—			NEGATIVE
D 5 Subpass to Broth		5.0	—	4/11/88	4/26/88	NEGATIVE
to Agar		.2	.2			NEGATIVE
D10 Subpass to Broth		5.0	—	4/15/88	5/2/88	NEGATIVE
to Agar		.2	.2			NEGATIVE
D15 Subpass to Broth		5.0	—	4/20/88	5/5/88	NEGATIVE
to Agar		.2	.2			NEGATIVE
Control Fluid - LOT # DENGUE-4 TCF-MYC# 015						
PPLO Agar		.2	.2	4/5/88	4/20/88	NEGATIVE
PPLO Broth		25.0	—			NEGATIVE
D 5 Subpass to Broth		25.0	—	4/11/88	4/26/88	NEGATIVE
to Agar		.2	.2			NEGATIVE
D10 Subpass to Broth		25.0	—	4/15/88	5/2/88	NEGATIVE
to Agar		.2	.2			NEGATIVE
D15 Subpass to Broth		25.0	—	4/20/88	5/5/88	NEGATIVE
to Agar		.2	.2			NEGATIVE

Positive Control (+): DV-2 Reagent Negative Control (-): FB 2910/C070

Date: 5/9/88 Signed: [signature]

BVL Ben Venue Laboratories, Inc.
est.1938

[illegible]

Dr. Louis Potash
Flow Labs. Inc.
7655 Old Springhouse Rd.
McLean, VA. 22101

Dear Dr. Potash,

This is to certify that Dengue Virus Type 4 (Non-Attenuated) Strain 341750 (Carib) Lot # 2, has been tested for Sterility and is found to be sterile according to USP XXI Specifications.

Enclosed is a copy of the test sheet for your files.
#58080 PF

Yours truly,
Ben Venue Laboratories Inc.

[illegible]

Encl.

270 Northfield Road, P.O. Box 46568, Bedford, Ohio 44146
Phone: 216-232-3320 Panafax: 216-232-2772

STERILITY TEST OF POWDERS
USP Membrane Filter Method

Date Sampled 4-6-88
Date Received 4-6-88
No. of Samples Received/Tested 10

BVL Control No. S 8 080 PF-
Product Dengue Virus Type 4 Non-Attenuated, Strain 341750 (Carib)
Lot No. 2
Thioglycollate No. L8056
Soybean-Casein Digest No. L8056
Date of Test 4/7 88
Operators Quirina McWilliams Moore.
N/A
Test Time 0900 to 1030

Sample Reconstituted with Sterile H_2O
Lot No. WS9104
Reconstituted Volume 3 mL
Type of Membrane Filter Used 0.2 um
Volume of Recon'd Sample Filtered 200 mL*
Volume of Fluid Thioglycollate 100 mL
Volume of Soybean-Casein Digest 100 mL
Volume of 0.1% Peptone Wash 600 (P8093) mL

	Thioglycollate	SCD
No. of Tubes used for Sterility Sample	1	1
No. of Reconstitution Fluid Controls	1	1
No. of Filter Controls	1	1
No. of Blank Media Controls	1	1
No. of Air Sham Media Controls	1	1
No. of 0.1% Peptone Wash Controls	1	1
No. of Tubes used for Water Control	NA	NA
No. of Tubes used for 250ml filter funnel controls	1	1

RESULTS:	Samples	Controls	Checked by
Date Read	4-26-88	4-26-88	C Barber
Fluid Thioglycollate (Present/Absent)	Absent	Absent	
No. of Tubes Contaminated	0	0	
Date Read	4-26-88	4-26-88	C Barber
Soybean-Casein Digest (Present/Absent)	Absent	Absent	
No. of Tubes Contaminated	0	0	

DENGUE VIRUS TYPE 4 (NON-ATTENUATED)

On the basis of the above data, STRAIN 341750 (CARIB), BVL Lot No. 2
Customer Lot No. 2 is sterile and is acceptable
as of 4-26-88.
Identification: N/A

Carol Barber
Bacteriologist/Senior Technician

Madeline Fuqua
~~Manager~~, Microbiology Department

COMMENTS: Steritest® * The vial contents pooled 30 ml. The pooled solution was diluted with 170 ml sterile H_2O for a total sample of 200 ml.

New
Revised X
Replaces 12/16/81
Date 4/24/85

REVERSE TRANSCRIPTASE ASSAY

SAMPLE	CPM INCORPORATED rAdT Mg	rAdT Mn	dAdT Mn
1. Dengue-4 TCF (Control fluid)	640	918	
2. Dengue-4 Virus (Challenge Seed, Lot 2)	1,085	1,085	
3. Hepatitis A TCF (control fluid)	318	2,371	
4. Hepatitis A Virus (FI2 Vaccine, Lot 1)	294	1,318	
CONTROLS			
50 µl growth medium	457	1,170	
50 µl culture fluid from cells infected with RSV	297,529	158,305	
10 µl culture fluid from cells infected with MLV + 40 µl growth medium	42,923	2,333,732	

Comments

All samples were negative for polymerase except sample #3. This sample contained a low but significant activity. It is likely that this activity is due to contaminating cell DNA dependent DNA polymerase from cell debris. Since this sample is a control fluid and the activity is not present in the vaccine, we have elected to not perform the dAdT assay to demonstrate this contamination. If you would like, we can perform this assay.

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